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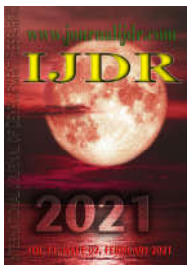

RESEARCH ARTICLE OPEN ACCESS

BLOODSTREAM INFECTION BY MULTIDRUG-RESISTANT *ENTEROBACTERIACEAE* IN MIDWEST BRAZIL: RISK FACTORS AND CLINICAL EVOLUTION

Camila Nogueira Espínola[1], Rodrigo Silva De Quadros[2], Nayara Ferreira da Silva[3], Ana Claudia Souza Rodrigues[4] and Marilene Rodrigues Chang[3,*]

[1]Faculty of Medicine, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil
[2]Beneficent Association of Campo Grande, Campo Grande, MS, Brazil
[3]Faculty of Pharmaceutical Sciences, Food and Nutrition, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil
[4]Faculty of Medicine, University Anhanguera Uniderp, Campo Grande, MS, Brazil

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Corresponding author:
Marilene Rodrigues Chang

ABSTRACT

This study describes resistance genes in *Enterobacteriaceae* isolates, risk factors and clinical evolution of 40 patients with bloodstream infection (BSI) from Intensive Care Units and Emergency Care in a tertiary hospital from Campo Grande, MS, Brazil. Clinical data was collected from medical records. Bacterial identification was performed by VITEK-2 system. Phenotypic detection of carbapenemases were screened by the Carbapenem Inactive Method. Bla_{KPC} and *mcr-1* genes were investigated by Polymerase Chain Reaction. Patients aged 60 years or over (15; 7.5%) and patients under one year (13; 32.5%) represented the majority. Twenty-five patients (62.5%) were admitted to the ICU for eight days or more. Seventeen patients died (42.5%). The length of stay in the ICU (p=0.028), use of central venous access (p=0.026) and mechanical ventilation (p=0.007) were associated with mortality. *Klebsiella pneumoniae* and *Echerichia coli* were the main bacteria isolated from blood cultures. Twenty-two (55%) were resistant to carbapenems, and four (10%) to polymyxin. Bla_{KPC} gene was detected in 37.5%. *Mcr-1* gene was not detected. Immune senescence, immature immune system and invasive procedures are considered risk factors for BSI. This infection caused by multidrug resistant enterobacteria is associated with elevated mortality.

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INTRODUCTION

Bloodstream infections (BSI) represent a serious health issue and one of the main complications in critically ill patients, especially in Intensive Care Units (ICU). These infections are multifactorial, therefore distinct methods of diagnosis and therapy are needed. Invasive procedures, which are frequent and necessary in ICU, are a crucial source for the acquisition of BSI (Burnham, Rojek & Kollef, 2018; Sante, Lecuona, Jaime & Arias, 2019). Carbapenem resistant enterobacteria (CRE) are among the most frequent agents responsible for BSI in hospitalized patients. In addition, CRE are an important risk factor for mortality, since carbapenems are broad-spectrum antibiotics considered drugs of choice for the treatment of serious infections (Diekema *et al*, 2019; Gupta, Limbago, Patel & Kallen, 2011). A common mechanism of resistance against carbapanems is the beta-lactamase *Klebsiella pneumoniae* carbapenemase (KPC), which have been reported in different *Enterobacteriaceae*, including *Klebsiella pneumoniae* and *Escherichia coli*. KPC is a widely distributed mechanism, once the encoding gene, the bla_{KPC}, can be transferred to other bacteria via plasmids and has great potential of dissemination (Chen *et al*, 2014). Polymyxins, such as colistin, are former antibiotics used to treat serious infections by enterobacteria. With the significant increase of multidrug resistance, colistin became a noticeable last option for treatment of serious BSI caused by CRE. However, new genes that induce resistance against colistin are emerging.

The first *mcr-1*report was published in 2016 and, ever since, this gene and its variants are fastly spreading globally (Liu *et al*, 2016; Sekyere, Maningi, Modipane & Mbelle, 2020; Wang *et al*, 2020). This study aimed to describe the clinical evolution of patients with bloodstream infection and to identify the bla_{KPC} and *mcr-1* genes, responsible for multidrug resistance.

MATERIALS AND METHODS

Patients: In this study, 40 patients with bloodstream infection caused by *Enterobacteriacea*, who attended a tertiary hospital from July to December of 2017, were analyzed. Two positive bloodstream samples from the same agent were considered as criteria for BSI. Patients with only a positive blood culture, patients transferred from other health institutions and unviability of bacterial samples to search for resistance genes were excluded from the study.

Data collection and studied variables: The clinical and demographic data and evolution of the participants were collected from the electronic hospital record. The studied variables were: age, sex, surgical procedures during hospitalization, length of stay at the ICU and Emergency Care, comorbidities, invasive procedures, antimicrobial therapy administrated and clinical evolution.

Bacterial identification: The selected microorganisms with their respective antibiogram reports were provided for analysis. The samples used for the tests were properly identified and stored at -20 ° C in BHI broth (Brain Heart Infusion) enriched with 15% glycerol. The identification of microorganisms was performed in the routine of the Hospital's Microbiology Laboratory, by an automated VITEK-2 compact system (bioMérieux, Marcy LÉtoile, France).

Detection of carbapenemases and antimicrobial resistance genes: Phenotypic investigation of carbapenemases was performed according to the Carbapenem Inactivation Method (CIM) technique, described by van der Zwaluw *et al* (2015). The result was considered positive for carbapenemase when there was no inhibition halo. The bacterial DNA for the Polymerase Chain Reaction (PCR) test was extracted by boiling method. The bacteria were individually suspended in sterile MiliQ water and heated in a dry bath at 90 ° C for 1 minute. Then, they were centrifuged at 12.000 rpm for 2 minutes. The supernatant was collected and used to perform the PCR. The resistance coding genes were investigated by simple PCR technique, using primers and reaction conditions described by Monteiro (2009) and Liu *et al* (2016). The bla_{KPC} gene investigation were performed using the primers sequence (Monteiro, 2009): *KPC F* 5′-TGTCACTGTATCGCCGTC-3′; *KPC R* 5′-CTCAGTGCTCTACAGAAAAACC-3′. The reaction conditions were: denaturation at 94 ° C for 5 minutes, followed by 30 cycles of denaturation at 94° C for 25 seconds, annealing at 56 °C for 40 seconds and extension at 72 °C for 50 seconds. After the cycles, a final extension step at 72 °C for 6 minutes. The mcr-1 gene investigation were performed sing the primers sequence (Liu *et al*, 2016): *MCR-1 F* 5′-CGGTCAGTCCGTTTGTTC-3′; *MCR -1 R* 5′-CTTGGTC GGTCTGTA GGG-3′. The reaction conditions were denaturation at 94 °C for 15 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 90 seconds and extension to 72 °C for 60 seconds. After the cycles, a final extension step at 72 °C for 10 minutes.

Statistical analysis: The association between the outcome of patients and the variables evaluated in this study was performed using the chi-square test. The other results of the variables were presented in the form of descriptive statistics or in the form of tables. The statistical analysis was performed using the statistical program SigmaPlot, version 12.5, considering a significance level of 5%.

Human subject protection: This study was approved by the Ethics Committee of Federal University of Mato Grosso do Sul (CAAE - 20621919.4.0000.0021).

RESULTS

Among the 40 patients studied, the majority of them (23; 57.5%) were male. The age ranged between 0 and 86 years, with an average age of 35.38 ± 5.28 years (mean ± standard error of the mean). Table 1 presents the demographic data, length of stay, invasive procedures, comorbidities and clinical evolution of the patients. The most common agents of BSI were *Klebsiella pneumoniae* (17; 42.5%) and *Escherichia coli* (12; 30.0%).

Table 1. Demographics, clinical characteristics and risk factors for bloodstream infection of 40 patients

	N (%)
Sex	
Male	23 (57.5)
Female	17 (42.5)
Age range	
0 to 11 months	13 (32.5)
1 to 18 years	5 (12.5)
19 to 59 years	7 (17.5)
Equal to or above 60 years	15 (37.5)
Length of ICU stay (days)	
0	6 (15.0)
1 to 7	9 (22.5)
8 to 30	15 (37.5)
More than 30	10 (25.0)
Length of stay in the Emergency Care (days)	
Until 7	36 (90.0)
8 to 30	2 (5.0)
More than 30	2 (5.0)
Central venous access	29 (72.5)
Surgical procedure	14 (35.0)
Mechanical ventilation	31 (77.5)
Bladder catheter	21 (52.5)
Thoracic drainage	7 (17.5)
Comorbidities	
Pneumonia	8 (20.0)
Renal insufficiency	7 (17.5)
Brain vascular accident	3 (7.5)
Death outcome	17 (42.5)

A total of 22 enterobacteria (55%) were ertapenem resistant and polymyxin resistance were observed in 4 enterobacteria (10%). Table 2 shows all the agents isolated.

Table 2. Enterobacteria isolated from blood culture of 40 patients

Enterobacteria	N (%)
Klebsiella pneumoniae	17 (42.5)
Escherichia coli	12 (30.0)
Enterobacter cloacae	9 (22.5)
Pseudomonas aeruginosa	1 (2.5)
Proteus mirabilis	1 (2.5)

In this study, the length of stay in the ICU, the use of central venous access and the treatment with mechanical ventilation were associated with a poor outcome ($p<0.05$).

Table 3 shows the relation between mortality and clinical conditions of patients. Fifteen (37.5%) of the enterobacteria demonstrated a positive results of carbapenemases on the phenotypic and genotypic tests. However, a divergence between the results of both tests was noticed. Two enterobacteria harboring the bla_{KPC} gene did not exhibit positive results for the presence of carbapenemases. In addition, two carbapenemase producing bacteria were tested negative for the bla_{KPC} gene. Figure 1 ilustrates the PCR-aplified produtcs for the bla_{KPC} gene (798 pb). The *mcr-1* gene was not detected among the enterobacteria studied. Table 4 presents the antimicrobial resistance profiles of KPC positive and KPC negative enterobacteria. Multidrug resistance (MDR) was considered when the enterobacteria was not susceptible to at least one agent in three or more categories of antimicrobials. Extensively resistance (XDR) were considered when the enterobacteria were susceptible to only one or two categories of antimicrobials (Magiorakos *et al*, 2012).

DISCUSSION

We analyzed the risk factors for bloodstream infection and the resistance profile of last choice antibiotics, in order to obtain a better understanding of the scenario in our region and to contribute with epidemiologic data for the public health institutions. Patients over 60 years old present immune senescence, whilst the immune system of patients under two years old is still immature. These characteristics cause patients to be more likely to develop BSI. In addition, staying in the ICU for a period longer than eight days is considered an important risk condition for the acquisition of bloodstream infections (Aw, Silva & Palmer, 2007; Simon, Hollander & McMichael, 2015). In ICU, patients are submitted to various invasive procedures necessary for their treatment and survival. Among these, central venous access is one of the main invasive procedures often used (O'Grady *et al*, 2011).

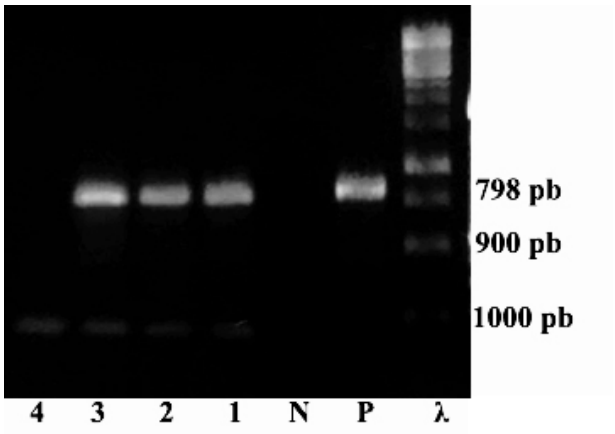
Table 3. Relationship between the risk conditions of 40 patients with BSI and mortality

Condition	Mortality			*p* value
	Deceased N (%)	Survivor N (%)	Not registered	
Length of ICU stay (days)				
0	3 (50.0)	3 (50.0)	0	0.028
1 to 7	8 (88.9)	1 (11.1)	0	
8 to 30	4 (28.6)	10 (71.4)	1	
More than 30	7 (70.0)	3 (30.0)	0	
Length of stay in the Emergency room (days)				
Until 7	20 (57.1)	15 (42.9)	1	0.963
8 to 30	1 (50.0)	1 (50.0)	0	
More than 30	1 (50.0)	1 (50.0)	0	
Central venous access				
No	7 (87.5)	1 (12.5)	0	0.026
Yes	12 (42.9)	16 (57.1)	1	
No information	3	0		
Surgical procedure				
No	9 (52.9)	8 (47.1)	1	0.524
Yes	9 (64.3)	5 (35.7)	0	
No information	4	4		
Mechanical ventilation				
No	7 (100.0)	0 (0.0)	0	0.007
Yes	13 (43.3)	17 (56.7)	1	
No information	2	0		
Pneumonia				
No	17 (54.8)	14 (45.2)	1	0.697
Yes	5 (62.5)	3 (37.5)	0	
Renal insufficiency				
No	18 (56.3)	14 (43.8)	1	0.966
Yes	4 (57.1)	3 (42.9)	0	
Brain vascular accident				
No	21 (58.3)	15 (41.7)	1	0.401
Yes	1 (33.3)	2 (66.7)	0	

Statistically significant = $p < 0.05$.

Table 4. Antimicrobial resistance profiles of KPC positive and KPC negative enterobacteria

Antibiotic	bla_{KPC}		Total
	Positive (n=15)	Negative (n=25)	(n=40)
	n° (%)	n° (%)	n° (%)
Amikacin	0 (0.0)	1 (4.0)	1 (2.5)
Cefepime	11 (73.3)	17 (68.0)	28 (70.0)
Ceftriaxone	12 (80.0)	16 (64.0)	28 (70.0)
Ciprofloxacin	9 (60.0)	10 (40.0)	19 (47.5)
Colistin	2 (13.3)	2 (8.0)	4 (10.0)
Ertapenem	8 (53.3)	15 (60.0)	22 (55.0)
Gentamicin	8 (53.3)	12 (48.0)	20 (50.0)
Imipenem	7 (46.7)	6 (24.0)	13 (32.5)
Meropenem	7 (46.7)	6 (24.0)	13 (32.5)
Piperacillin/Tazobactam	9 (60.0)	14 (56.0)	23 (57.5)
Tigecycline	11 (73.3)	2 (8.0)	4 (10,0)
Multidroga resistente - MDR	13 (86.7)	17 (68.0)	30 (75.0)
Extensivamente resistente - XDR	4 (26.7)	2 (8.0)	6 (15.0)



Lane λ: molecular weight marker; P: positive control; N: negative control; Lanes 1-3: positive samples; Lane 4: negative sample.

Figure 1. Agarose gel electrophoresis of the PCR amplification products for the *bla*$_{KPC}$ gene

In this study, the majority (72.5%) of the patients used central venous access, considered a significant risk factor for the development of BSI (Bell & O'Grady, 2017; Ruiz-Giardin *et al*, 2019). According to previous studies, one third of deaths in health facilities in the United States are due to blood infections associated with the use of central venous catheters (CVC) (Burnham, Rojek and Kollef, 2018). In this study, this device was indicated as an important risk factor for mortality. These results suggest that care in inserting, maintaining and removing CVC need to be intensified. Mechanical ventilation (MV), another frequent (77.5%) invasive procedure also used by the patients under intensive care, was considered a risk factor for mortality. Literature data show that MV is a risk condition for hospital pneumonia, which may evolve to secondary bloodstream infection (Oliveira, Zagalo, Cavaco-silva, 2014).

Surgical procedures facilitate the translocation of endogenous bacteria into the bloodstream. Many BSI bacterial agents are part of the human microbiome. Any interruption in the balance of microbiota or immune system can facilitate infections (Bai *et al*, 2019). It is possible that Escherichia coli and Klebsiella pneumoniae isolated as an agent of bloodstream infection are of endogenous origin, since 35% of patients have undergone previous surgery. Bloodstream infections can be caused by Gram-positive bacteria (mainly *Staphylococcus* spp.) and Gram-negative bacteria (mainly *Enterobacteriaceae* and non-fermenting Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter* spp.) (Bassetti, Righi & Carnelutti, 2016). In this study, we selected enterobacteria due to the great number of bacteria resistant to antimicrobials in the hospital. The number of enterobacteria considered XDR is alarming, taking into consideration that 15% were sensitive to only one or two classes of antibiotics. Therefore, the lack of drugs available for treatment, combined with the use of invasive devices, may have influenced the high rate of death (42.5%) reported in this study.

Although in vitro susceptibility corresponds to the in vivo situation, it is known that when the infection is caused by multidrug resistant bacteria, the clinical outcome tends to be unfavorable (Manyahi, Kibwana, Mgimba & Majigo, 2020). In this regard, it is of great relevance for every hospital institution to know the profile of resistance to antimicrobials by infection sites.

This resistance profile can vary from one institution to another (Campos *et al*, 2017). The Microbiology laboratory must provide this information to collaborate with the treatment and for the purposes of epidemiological studies. Many tools are available for detecting carbapenem-resistant bacteria. The most simple and low cost are those of phenotypic tests (such as Modified Hodge Test and the Carbapenem Inactivation Method) and those that use advanced technology (molecular methods) (Clinical and Laboratory Standards Institute, 2018; van der Zwaluw *et al*, 2015).

In this study, when comparing the two methodologies for the detection of carbapenemases, we observed a good correlation between the MIC method and the PCR (84% of specificity and 73% of sensitivity). These results suggest that this phenotypic method can be used to screen for the presence of carbapenemases in the laboratory routine, seeing that it is a cheap and fast technique. The fact that 55% of enterobacteria studied are carbapenem resistant and 37.5% expressed the *bla*$_{KPC}$ gene suggests that other mechanisms than KPC are involved in the carbapenem resistance in this hospital.

However, we notice that two *bla*$_{KPC}$ gene-carrying enterobacteria had CIM test negative, suggesting a false negative. According to Pierce *et al* (2017), the sensitivity of the CIM method may improve by replacing water for trypticase soy broth. Corroborating with Rodrigues *et al* (2019), the PCR results show that *mcr-1* gene is not responsible for the resistance observed to colistin and other resistance mechanisms are involved. Rodrigues *et al* (2019) reported that the resistance to colistin by *K. pneumoniae* isolated in the same hospital was mainly due to deleterious mutations in the *pmrB* gene. This study confirms that the *mcr-1* is not present in Mato Grosso do Sul state, Brazil. However, it was already found in clinical samples, animal samples and environmental samples from the South, Southwest and Northwest regions of Brazil (Fernandes *et al*, 2017; Kieffer *et al*, 2018; Lorenzoni, Dalmolin, Franco, Barth & Hörner, 2018; Vasconcelos, 2020). Indiscriminate use of antibiotics is indicated as one of the factors for bacterial multidrug resistance. Inadequate therapy is considered a risk factor for poor prognosis among patients infected with MDR enterobacteria (Santoro *et al*, 2020). Although only one (2.5%) enterobacteria showed resistance to amikacin, this drug is not indicated for monotherapy, since aminoglycosides have toxic properties that limit their administration and time of treatment (Block & Blanchard, 2020). However, the combined therapy of an aminoglycoside with tigecycline and meropenem can be an option for BSI, resulting in a reduced mortality rate compared to the monotherapy (Yamamoto & Pop-Vicas, 2014). The main limitations of this study were that due to the retrospective data collection, some clinical data were incomplete. Limitations like this have already been described by different researchers (Ting, Lee & Liu, 2017).

CONCLUSION

Our results show that immune senescence and immature immune system are important risk factors for que acquisition of bloodstream infection by enterobacteria. Prolonged length of hospital stay and the use of central venous access and mechanical ventilation are risk conditions for mortality among patients with BSI by enterobacteria. *Klebsiella pneumoniae* carbapenemase is an important mechanism of resistance against carbapenem antibiotics in Enterobacteriaceae isolated

from blood culture in the studied hospital. On the other hand, resistance to colistin is not associated with the *mcr-1* gene. BSI caused by multidrug resistant enterobacteria are associated with elevated mortality.

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